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Co-cultures of multiple cell types mimic pulmonary cell communication in response to urban PM_{10}

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Short title:

Multiple co-cultures response to PM_{10} exposure

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Abstract

We evaluated if a system of co-cultures of relevant cells [pneumocytes (A549), macrophages (THP-1), mast cells (HMC-1) and endothelial cells (EAHY926)] mimics responses to PM_{10} previously reported *in vivo*. The role of mast cells was considered of special interest.

Single cultures, BICULTURES (A549 + HMC-1 @ 10:1 ratio; THP-1 + HMC-1 @ 2:1 ratio) and TRICULTURES (A549 + THP-1 + HMC-1 @ 10:2:1 ratio) were exposed to urban PM_{10} (24 h @ 0, 10, 30 or 100 μg/cm^2). In further experiments, EAHY926 cells were introduced in inserts above the TRICULTURES. The released cytokines were evaluated with a FACS array system.

THP-1 + HMC-1 BICULTURES and the TRICULTURES released more G-CSF, MIP-1β, IL-1β, IL-8, IL-6, TNFα, and MIP-1α in response to PM, than the sum of the single cultures. TRICULTURES + EAHY926 released more G-CSF, MIP-1α, IL-8 and MIP-1β than the EAHY926 single culture.

The BICULTURES, TRICULTURES and TRICULTURES + EAHY926, provide results that are consistent with the local and systemic effects previously described for PM effects, i.e. inflammation, endothelial dysfunction and bone marrow cell mobilization. Mast cells seem to play a significant role in the co-cultures responses.

Keywords: co-culture, cytokine profile, PM_{10}, Tricultures.

Introduction

In vitro and *in vivo* studies have evaluated the proinflammatory effects of particulate matter (PM) [1,2], but the role of communication between different pulmonary cell types is not well known. In this regard, Ishii et al. showed that the interaction between bronchial epithelial cells and macrophages led to an enhanced response to PM, and that this interaction was independent of cellular cross-linking [3,4].

Considering the complex architecture of airways and alveoli, the use of one and even two cell types is a far cry from reality. The interactions of epithelial cells and macrophages, i.e. the cells having direct contact with deposited particles, with other important cell types such as mast cells, dendritic cells, fibroblasts and endothelial cells, among others, ought to be important but this remains largely unknown. In the present study we focused on the role of mast cells in the response to PM and the possible interaction of these cells with macrophages and epithelial cells, and the further interaction of co-cultures of these three cell types with endothelial cells.

Mast cells are an important component of the bronchial submucosa, the pleura and even the alveolar septa [5,6]. Mast cells have not been studied much in relation to PM. However, histamine has been demonstrated to play an important role in the systemic effects of PM [7-9], and in their translocation [10], and considering that histamine is mainly secreted by mast cells, it seems that mast cells may be crucial to understand the systemic effects of PM. So, we evaluated the release of cytokines in response to PM, obtained in Mexico City, by single cultures of mast cells, pneumocytes, macrophages and endothelial cells; and then by co-cultures of mast cells with pneumocytes or macrophages (BICULTURES); as well as co-cultures involving the three cell types (TRICULTURE), and finally the interaction of these TRICULTURES with endothelial cells. The hypothesis was that the release of cytokines would differ qualitatively and quantitatively between pure cell cultures and mixed cell cultures.

Methods

Protocol design

In order to evaluate the interactions and contributions of different cell types in the lung, we created a co-culture system with up to four different cell types. We first studied single cultures of lung epithelial cells (A549), macrophages (THP-1), mast cells (HMC-1) and endothelial cells (EAHY926) (see further). Then, we prepared co-cultures of two, three and four different cells: BICULTURES of A549 with HMC-1 (10 to 1 ratio), and of THP-1 with HMC-1 (2 to 1 ratio); TRICULTURES of A549, THP-1 and HMC-1 (10:2:1 ratio); TRICULTURES plus EAHY926 introduced in an insert after 12 h of exposure of the TRICULTURES (Figure 1). The different cultures were exposed to PM_{10} collected in Mexico City.

Particulate matter sampling and preparation

PM_{10} was collected using a high volume sampler (GMW Model 1200, VFC HVPM10; Sierra Andersen, Smyrna, GA, USA), in the industrial region of the Metropolitan Zone of Mexico City, in Xalostoc. Samples were collected during 24 h with an airflow rate of 1.13 m^3/min ± 10% on cellulose nitrate membranes with a nominal pore-size of 3 μm (11302-131, Sartorius, Goettingen, Germany). Sampling was done three times a week, from November 2004 until April 2005.

The recovered particles were suspended in the required cell culture medium (see further) at a final concentration of 1 mg/mL. The suspended particles were sonicated during 20 minutes to avoid the presence of particle-clusters. Aliquots were taken to get the necessary final concentrations. The final exposure concentrations are expressed in μg/cm^2 in order to keep a consistent ratio of particulate mass / cell-number / area [11]

Cell culture

The following human-derived cell lines were used: A549 (epithelial type II pneumocytes) (American Type Culture Collection, Manassas, VA, USA) [12]; THP-1, a monocyte-derived cell line (American Type Culture Collection, Manassas, VA, USA) differentiated into macrophage-like cells by overnight incubation with 1 ng/mL phorbol myristate acetate (PMA) [13], HMC-1 (mast cells) (kindly provided by Dr J.H. Butterfield, Mayo Clinic, Rochester, MN, USA) [14]; and EAHY926 (endothelial cells) [15]. The cell culture media used for each different culture are described in table 1. All the experiments were performed in polystyrene 24-well plates (Costar) seeded at a density of 1.6 x 10^5 cells/cm^2. The EAHY926 cells were grown on polycarbonate Transwell inserts with a nominal pore size of 0.4 µm (Costar). After 24 h, the medium was changed for medium without fetal calf serum (FCS).

To our knowledge, there is no report on the number of mast cells in the lung, so we chose a ratio of 10:1 for the epithelial cells and mast cells BICULTURE (1.6 x 10^5 epithelial cells / cm^2 and 0.16 x 10^5 mast cells /cm^2), and a 2:1 ratio for the macrophages (1.07 x 10^5cells/cm^2) and mast cells (0.53x 10^5 cells/cm^2) BICULTURE.

Based on the evaluation of their *in vivo* distributions made by Stone et al., [16] a ratio 5:1 was chosen when co-cultures of epithelial cells and macrophages were used. The TRICULTURES of A549+THP-1+HMC-1 cells were seeded in a ratio of 10:2:1, i.e. 1.6 x 10^5, 0.32 x 10^5, 0.16 x 10^5 cells/cm^2, respectively (Figure 1). The TRICULTURES + Endothelial cells consisted of adding an insert containing confluent EAHY926 cells, thus creating another compartment, above the TRICULTURES after 12 h of exposure to PM. Thus, the EAHY926 cells were never in direct contact with the PM (Figure 1).

All incubations with PM were performed with FCS-free medium. The cell culture medium was changed for a FCS-free medium 6 h before exposure to PM. The cultures were exposed to 0, 10, 30 or 100 µg/cm^2 of PM_{10} and after 24 h the supernatants were recovered and stored at –80^{o}C for further analyses.

Cytokines release

Qualitative analysis

In preliminary experiments, supernatants from the single cultures, the TRICULTURES and TRICULTURES+endothelial cells exposed to 0 or 100 µg/cm^2 were evaluated to establish a cytokine secretion profile by a semiquantitative technique (Proteome Profiler, Human Cytokine Array Kit, R&D). The obtained autoradiographs were scanned and analyzed using the J-image program (NIH). The relative density of each dot was calculated in relation to the positive internal controls of the membranes and. The results were expressed as "fold" above or below the unexposed cultures. Changes of less than 0.5 or more than 1.5 were considered relevant. Those cytokines presenting a "relevant" change were selected for further analysis and quantification.

Quantitative analysis

Based on the results of the Human Cytokine Array, TNFα, interleukin (IL)-1β, IL-6, IL-8, MIP-1α, MIP-1β, MCP-1, G-CSF, IFNγ and RANTES were selected for quantitative analysis using a Cytometric Bead Array Flex (BD Biosciences, Erembodegem, Belgium), acquired with the FACSArray (BD Biosciences, Erembodegem, Belgium) in 50 µL of the supernatants using the FCAP Array software (BD Biosciences, Erembodegem, Belgium).

Statistical analysis

Due to the variability of the cytokine release in the different cell culture conditions, the results are presented as percentages of the concentrations in the unexposed cultures. To evaluate how the concentrations of cytokines in the co-cultures (and to put these results into perspective), we also compared the results from the co-cultures with the "expected"

concentrations obtained by adding up the amounts secreted in the single cultures, adjusting for the number of cells. Database management and statistical analyses were done with SAS software (version 9.1). We looked for differences across the three PM concentrations by analysis of covariance. For testing the effect of PM on co-cultures, data from the single cultures, adjusted by cellular density, were used to calculate an expected additive effect for each concentration. A possible multiplicative effect at the different concentrations was studied by two-way ANOVA with interaction testing between the additive data and the observed data. Differences were considered significant when $p < 0.05$ (two-tailed).

Results

Qualitative analysis (Cytokine array)

Supernatants collected from single cultures evaluated by the Proteome Profiler Human Array kit (Table 2, Figure 2) showed that A549 cells exposed to PM exhibited a large decrease in the levels of IL-8 (0.27x), GROα (0.25x) and IL-23 (0.3x), while no relevant increase was observed for any cytokine. In contrast, the THP-1 cells exposed to PM exhibited increases in MIP-1β (1.5x), RANTES (1.5x), sICAM (2x) and TNFα (90x), and decreases in IL-8 (0.23x) and MIP-1α (0.5x). HMC-1 cells presented an increase in the secretion of IL-1β (3.5x) and sICAM (2.7x) but decreases in the levels of I-309 (0.35x), IL-32 (0.11x) and MCP-1 (0.05x).

In the TRICULTURE, increases were observed for G-CSF (4.4x), sICAM (1.5x), IL-1β (2.6x), IL-6 (4.4x), MIP-1α (8.4x), MIP-1β (13.4x) and a decrease in RANTES (0.32x). The supernatants from the apical compartment of the endothelial cells exhibited increases in the levels of G-CSF (22.5 x), and MIP-1α (65 x), but decreases for GROα (5x), I-309 (0.07x), MCP-1 (0.04x), and RANTES (0.1x).

Quantitative analysis

Single cultures (Figure 3)

A549 cells exposed to PM_{10} (Figure 3A and 3B) did not show an increase in any evaluated cytokine, but significant concentration-related reductions in the secretion of IL-8, MCP-1 and RANTES (Table 3).

THP-1 cells exposed to PM_{10} (Figure 3C and 3D) exhibited increases above 4 fold in the levels of TNFα and IFNγ and decreases in the levels of MCP-1. IL-8 had a slight increase at a

low concentration of PM_{10} (10 µg/cm^2) and a decrease at high concentration (100 µg/cm^2) (Table 3).

For the HMC-1 cells exposed to PM_{10} (Figure 3E and 3F) no increase was observed for any of the evaluated cytokines, and significant decreases were observed for IL-6, TNFα, MIP-1α and MCP-1 (Table 3).

BICULTURES (Figure 4 A-B)

In the BICULTURES of A549+HMC-1 cells exposed to PM_{10}, no significant increase was observed for any of the evaluated cytokines, but decreases up to 90 % were observed for IL-1β, IL-8 and MCP-1 (Figure 4A, 4B and Table 3). The co-cultures of THP-1+HMC-1 (macrophages & mast cells) induced the most significant changes (Figure 4C, 4D and Table 3). Large increases were observed for G-CSF (more than 10 fold), MIP-1α, MIP-1β, IL-1β (up to 8 fold), IL-6 and TNFα (up to 100 fold). In contrast, decreases were observed for the levels of RANTES and MCP-1. IL-8 exhibited increases at 10 and 30 µg/cm^2 but a return to basal levels at 100 µg/cm^2.

TRICULTURES (Figure 4E and 4F)

In TRICULTURES exposed to PM_{10} significant increases were observed for G-CSF (up to 6 fold), IL-1β, IL-6 (up to 7.5 fold), TNFα (up to 20 fold), and MIP-1α. An increase followed by a decrease compared to basal levels was observed for MIP-1β, IL-8 and MCP-1. A significant decrease was observed for RANTES (Table 3).

TRICULTURES + endothelial cells (Figure 4G and 4H) and endothelial cells alone (4I and 4J)

The supernatants from the endothelial cells co-cultured with the TRICULTURES exposed to PM_{10} presented significant increases in IL-6 (up to 7.5 fold), MIP-1β, IL-8 and MCP-1, but a decrease in RANTES (Figure 4G, 4H and Table 3). The endothelial cells exposed only to the medium with PM_{10} in the basolateral compartment exhibited increases in the levels of TNFα up to 12 fold and G-CSF, and no significant variations in all the other mediators (Figure 4I, 4J and Table 3).

Observed versus expected cytokine concentrations:

In figure 5 (Figure 5) we present the results of the concentrations of TNFα, G-CSF and IL-8 in THP-1 + HMC-1, TRICULTURES and TRICULTURES + endothelial cells after exposure to PM_{10}, compared to the expected concentrations, based on the single cultures and corrected for the cell number.

Discussion

So far, the use of conditioned medium and co-cultures of macrophages plus epithelial cells have been useful for the evaluation of PM-induced effects [4,17,18], but with the present approach we added two more players: mast cells and endothelial cells. The use of multiple cellular types, relevant for the local and systemic effects attributed to PM, shows that regulation of cytokine secretion in single cell cultures can be amplified/mitigated in co-cultures. The pattern of cytokine secretion after exposure to PM appears to correspond to the *in vivo* effects related to PM. The most important and novel observation in our *in vitro* system is that the interaction between mast cells and macrophages leads to an amplified response to PM. These amplifications may mimic what really occurs in the lung especially in lungs from allergic subjects. The most significant increases in cytokines were observed in the THP-1 + HMC-1 BICULTURES and in the TRICULTURES.

Cytokine levels

Although cytokines can share more than one function, we categorized, for the sake of simplicity, the mediators measured in our study into cytokines (TNFα, IL-6, IL-1β, IFNγ, G-CSF) and chemokines (IL-8, MCP-1, MIP-1β, MIP-1α, RANTES) [19]

After PM exposure, large changes were observed in cytokine secretion in the THP-1 + HMC-1 BICULTURE (TNFα increased more than 100 fold, G-CSF about 10 fold and IL-8 increased more than 5 fold): In the TRICULTURES and TRICULTURES + endothelial cells strong responses were observed, although they were not as strong as for the BICULTURE of THP-1 + HMC-1. This difference could be due to: 1) the amount of HMC-1 and THP-1 cells being 3.5 fold smaller in the TRICULTURE than in the BICULTURE, and/or 2) the presence of receptors in the A549 cells which could trap part of the secreted cytokines. The single cultures and the A549 + HMC-1 BICULTURE exposed to PM released significantly lower amounts of cytokines.

It is remarkable that PM in the basolateral compartment of endothelial cell cultures, induced a large increase of TNFα and RANTES detectable in the apical compartment, but no significant effect on any of the other mediators. In contrast, when the endothelial cells were introduced above the TRICULTURE already exposed during 12 h to PM, a large increase in the levels of most of the cytokines was observed in the apical compartment, but now without increases in TNFα or RANTES levels. This indicates that the interactions between different cells change the secretion profiles. Probably, this is attributable to cross-talk between the different cells, as already shown by others [20], or to the presence of receptors that capture some cytokines avoiding further detection in culture supernatants [21].

Cellular interaction

The multiple cell cultures represent an improvement compared to single cell cultures and resemble more closely the *in vivo* situation, yet it is also limited to the cells investigated. Macrophages, abundantly present in healthy individuals, in combination with structural cells (epithelial cells) are the main targets of PM. Consequently, this system mimics the very first interaction with PM found in the *in vivo* situation. The finding that the presence of mast cells increases the production of cytokines fits with the epidemiologic findings that air pollution aggravates the presentation of asthma [22]. The histamine secretion by the mast cell is considered to be low, because under similar experimental conditions, we have previously observed that PM only induces the secretion of histamine by HMC-1 cells in the presence of an ionophore [23]. It is, however, likely that other mediators, such as enzymes, are released besides histamine.

In vitro observations are consistent with in vivo effects

Among the pulmonary and systemic effects induced by PM, the increased expression of various cytokines has been described. For instance, TNFα, IL-6 and IL-8 are correlated to pulmonary inflammatory processes after exposure to PM [24,25]. In relation to the systemic effects, GM-CSF and G-CSF are related to the mobilization of cells from the bone marrow [4], IL-1β, IL-6 and TNFα are related to blood clot formation [26-28], and TNFα, IL-6 and IL-8 play a role in endothelial dysfunction [29,30]. In our system, TNFα, G-CSF and IL-8 are good examples of these cellular mediators.

TNFα leads to the expression of adhesion molecules involved in recruiting inflammatory cells [29,30]. High concentrations of TNFα in BICULTURES of THP-1 + HMC-1 exposed to PM were measured when compared to the expected concentrations based on the single culture (Figure 5A). These high concentrations were not observed when epithelial cells were present, on the contrary, significant decreases were found. This reduction of TNFα in the TRICULTURES and the apical side of the endothelial cells reflects possibly the binding of TNFα to the abundant A549.

The secretion of G-CSF, a molecule related to the mobilization of leukocytes from the bone marrow [31], showed a secretion 8 times larger in the TRICULTURE + EAHY926 cells than for the EAHY926 cells by themselves. In figure 5D to F it is shown that the THP-1 + HMC-1 BICULTURE did not secrete higher concentrations of G-CSF than expected from the single cultures, but the concentrations of G-CSF in the TRICULTURES and TRICULTURES + EAHY926 were significantly larger than expected, especially at the apical side of the TRICULTURE + EAHY926. The increases in G-CSF concentrations followed a linear concentration-response pattern. We assume that the concentration of G-CSF observed on the apical side is released by the endothelial cells and not from the translocation of the molecule from the basolateral

compartment. This strengthens the hypothesis that our system mimics the in vivo signal that triggers G-CSF secretion related to systemic effects as a result of PM exposure.

IL-8 is an important pro-inflammatory cytokine, related to the recruitment of neutrophils. Controversial results have been published so far, as some authors reported increases in IL-8 secretion after PM exposure [32,33] while others reported decreases in the same cytokine [20,34]. A study of respiratory damage in children chronically exposed to urban pollution in Mexico City, showed that serum levels of IL-8 were decreased when compared to children living in a cleaner environment [35]. The down-regulation of IL-8 has been related to different factors such as relatively late sampling [36], the presence of IL-4 and IL-10 [37], and soluble receptors of TNFα or CD14 [38,39]. In the present study, we observed a decrease in the IL-8 secretion by A549 cells and by the BICULTURE of A549 + HMC-1, but a large increase in the HMC-1 + THP-1 BICULTURE, the TRICULTURE and the TRICULTURE + EAHY926 at concentrations of 10 and 30 μg/cm^2, followed by a decrease at 100 μg/cm^2. The controversial results of IL-8 by different authors may be associated to dose, time of exposure, type of cell exposed and interactions between different cell types. We cannot rule out the possibility of cytokines binding to PM, as has been previously demonstrated [40].

In vivo and *in vitro* studies have shown that particulate matter is capable of inducing an endothelial dysfunction phenotype [29,41,42] and prothrombotic effects [8,43]. The translocation of PM into the bloodstream has been considered as a possible mechanism of these effects [44] and dendritic cells may play a role in the translocation of PM [45], but considering that PM translocation is probably a relatively limited phenomenon, the communication of cells having a primary contact with particles, with endothelial cells may play a more prominent role in the systemic effects of PM. In this study we provide indirect evidence of endothelial cell activation by the TRICULTURE system previously exposed to the PM.

The existing evidence in humans demonstrates that endothelial dysfunction and cardiovascular effects are related to PM exposure (46,47), in animals PM exposure has been linked to thrombogenesis, endothelium dysfunction and PM translocation, (10,28,42). The system presented in this study is a model, which can be used in addition to animal models, including knock-out and knock-in animals, and human studies (43). Due to its relative simplicity (compared to *in vivo*) it opens the opportunity of exploring in detail the mechanisms related to the local and systemic effects of particulate matter. One possibility is the use of monoclonal antibodies anti-cytokines or inhibitors of cytokine receptors to block a signal and, therefore, understand how cytokine secretion and cellular interactions are regulated (48).

In conclusion, we developed a novel *in vitro* system that mimics cell communication within the lung, thus leading to a better understanding of the different cellular mechanisms related to the responses after PM exposure, or any other contaminant such as endotoxin or transition metals. The BICULTURES, TRICULTURES and TRICULTURES + endothelial cells, provide results that are consistent with the local and systemic effects, such as inflammation, endothelial dysfunction and bone marrow cell mobilization, that have been described for PM. This novel system opens the possibility of using specific inhibitors for cytokines and transduction signals leading to a better understanding of the mechanisms related to the effects of environmental pollutants.

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Legends:

Figure 1

Schematic representation of the strategy of PM exposure with TRICULTURES, TRICULTURES + EAHY926 and EAHY926 and supernatants collection for further cytokine evaluations.

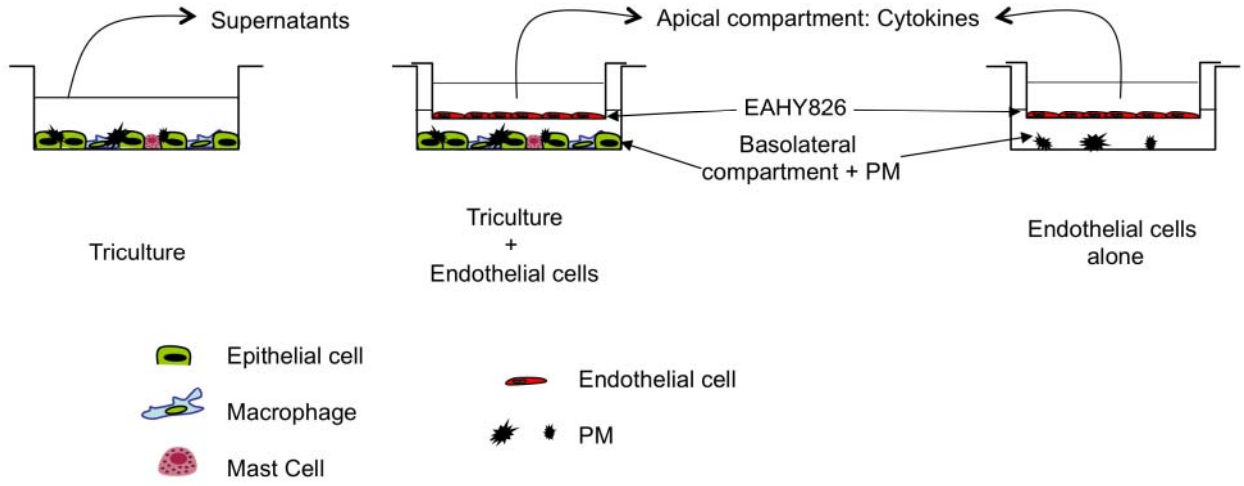

Figure 2

Protein array presenting the relative changes of the cytokine concentrations in medium of TRICULTURES exposed for 24 h to 0 or 100 µg/cm^2 of PM_{10}. The green and red boxes point to examples of proteins that are up-regulated (G-CSF and MIP-1β) or down-regulated (RANTES). The internal positive controls are marked with black boxes. The relative density of each dot was calculated in relation to the positive internal controls and the change in the intensity of the signal was calculated as a ratio between exposed and unexposed cultures and expressed as percentage change. The change was considered relevant when larger than 50% (see table 2).

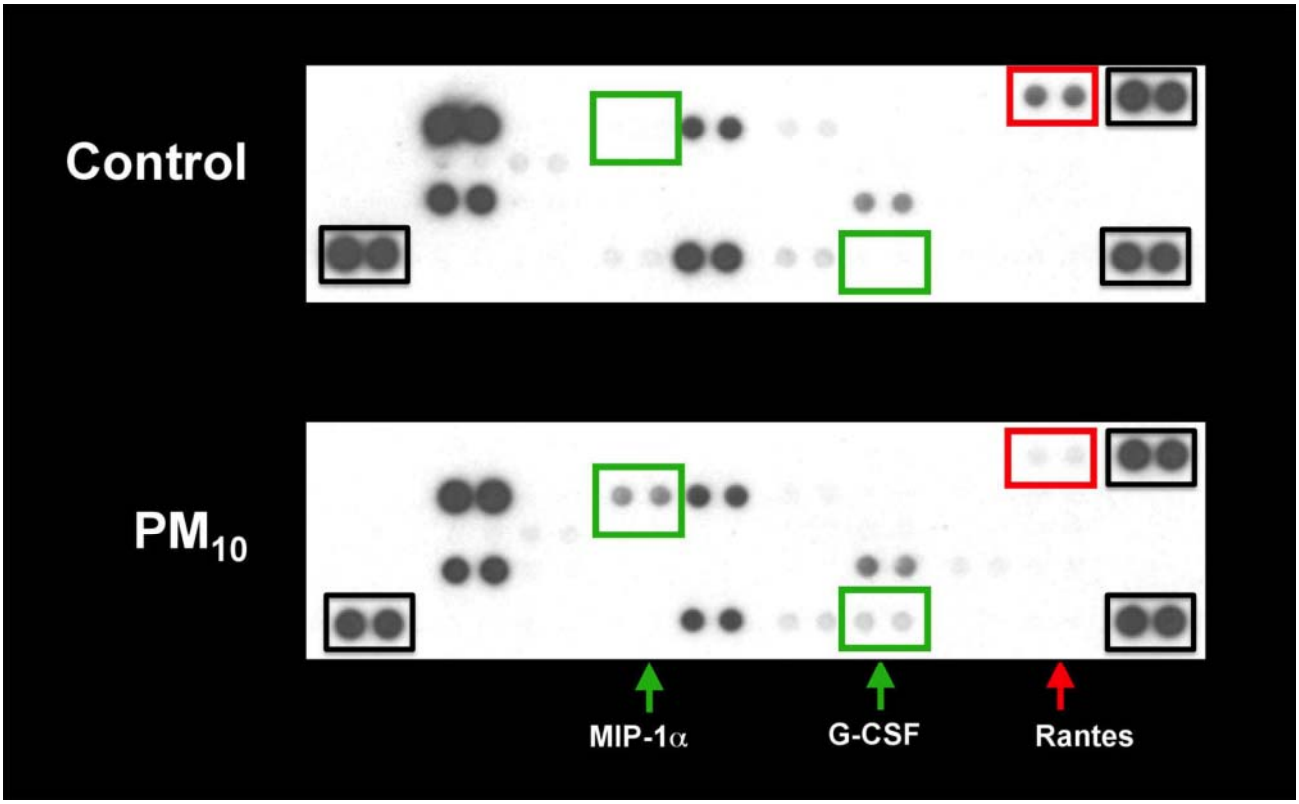

Figure 3

Cytokine secretion profile in cultures of A549 (A), THP-1 (B) or HMC-1 (C) exposed for 24 h to 0, 10, 30 or 100 µg/cm^2 of PM_{10} (Mean ± SD; n = 3). The cytokine levels are expressed as percentage of control.

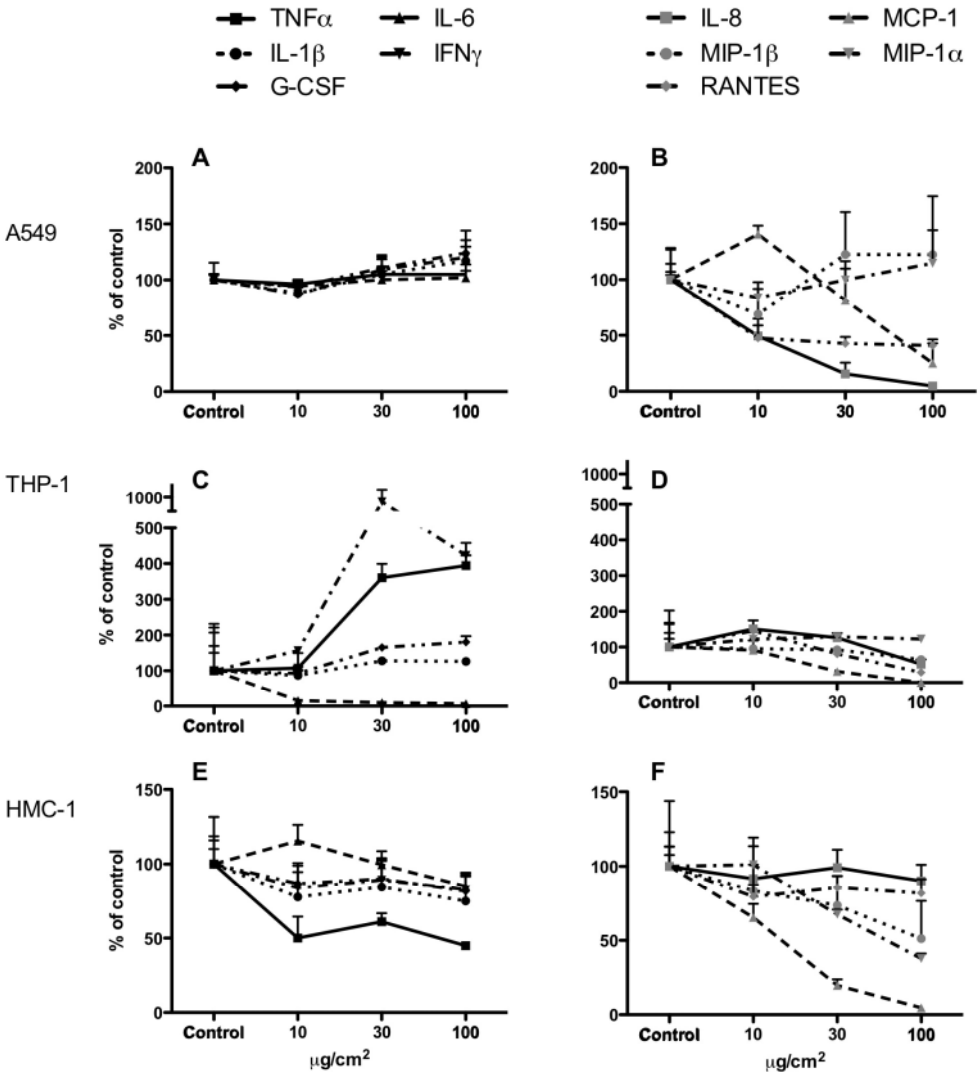
TNFα
IL-6
IL-1β
IFNγ
G-CSF
IL-8
MCP-1
MIP-1β
MIP-1α
RANTES
A549
THP-1
HMC-1
A
B
C
D
E
F
% of control
Control
10
30
100
μg/cm²

Figure 3

Figure 4

Cytokine secretion profile in BICULTURES of A549+HMC-1 (A) THP-1+HMC-1 (B), TRICULTURES (C), TRICULTURES + EAHY926 (D) or EAHY926 cells (E) exposed for 24 h to 0, 10, 30 or 100 µg/cm^2 of PM_{10} (Mean ± SD; n = 3). The cytokine levels are expressed as percentage of control.

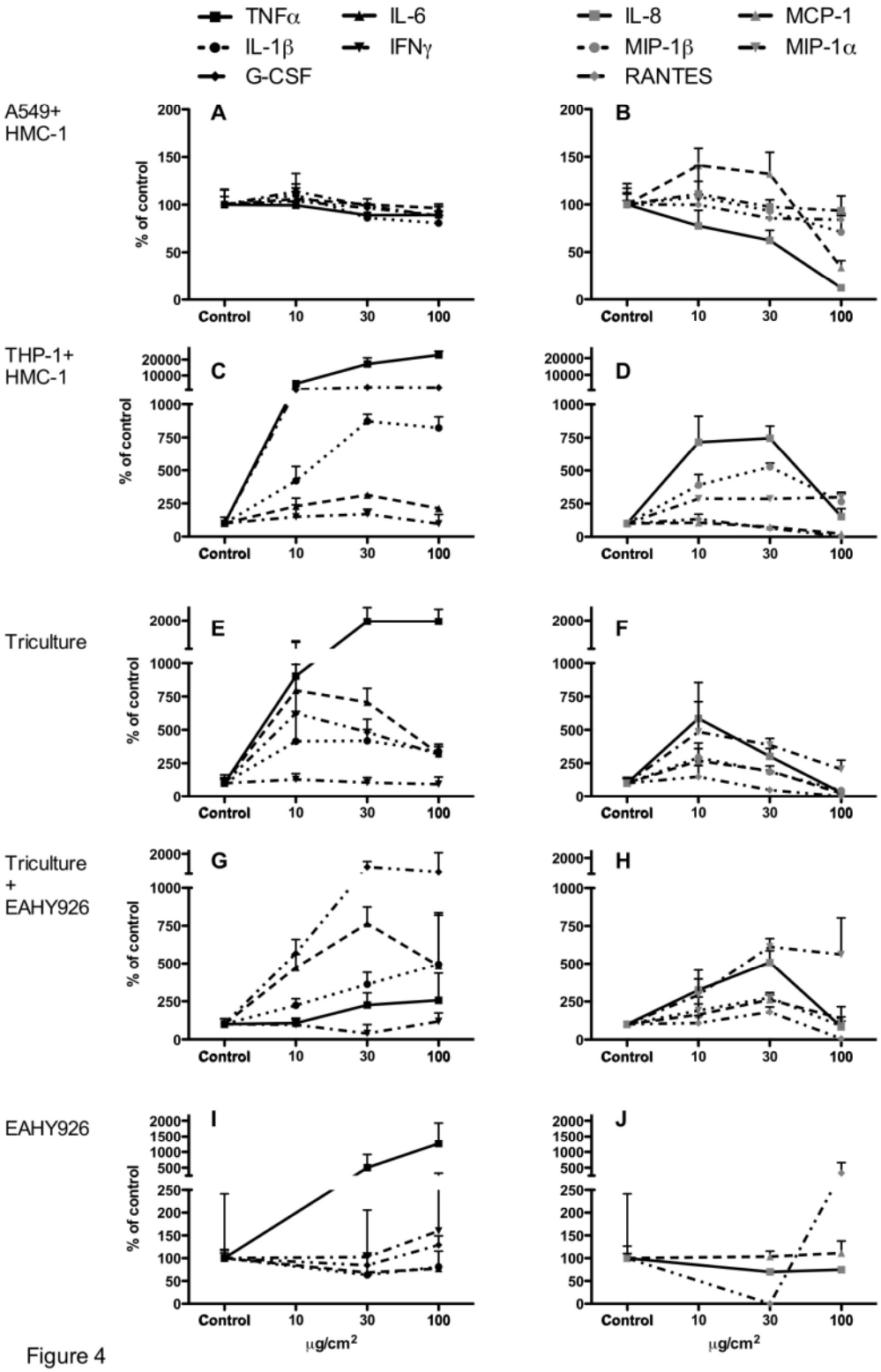
TNFα
IL-6
IL-1β
IFNγ
G-CSF
IL-8
MCP-1
MIP-1β
MIP-1α
RANTES
A549+
HMC-1
THP-1+
HMC-1
Triculture
Triculture
+
EAHY926
EAHY926
A
B
C
D
E
F
G
H
I
J
% of control
Control
10
30
100
μg/cm²

Figure 4

Figure 5

Comparison of the observed cytokine concentrations (pg/mL) vs. the expected concentrations (obtained by adding up the amounts secreted in the single cultures, adjusting for cellular density). TNFα(A, to C), G-CSF (D to F), and IL-8 (G to I), in THP-1 + HMC-1 BICULTURE (A, D and G), TRICULTURES (B, E and H) and TRICULTURE + EAHY926 cells (C, F, and I) (Mean ± SD; n = 3).

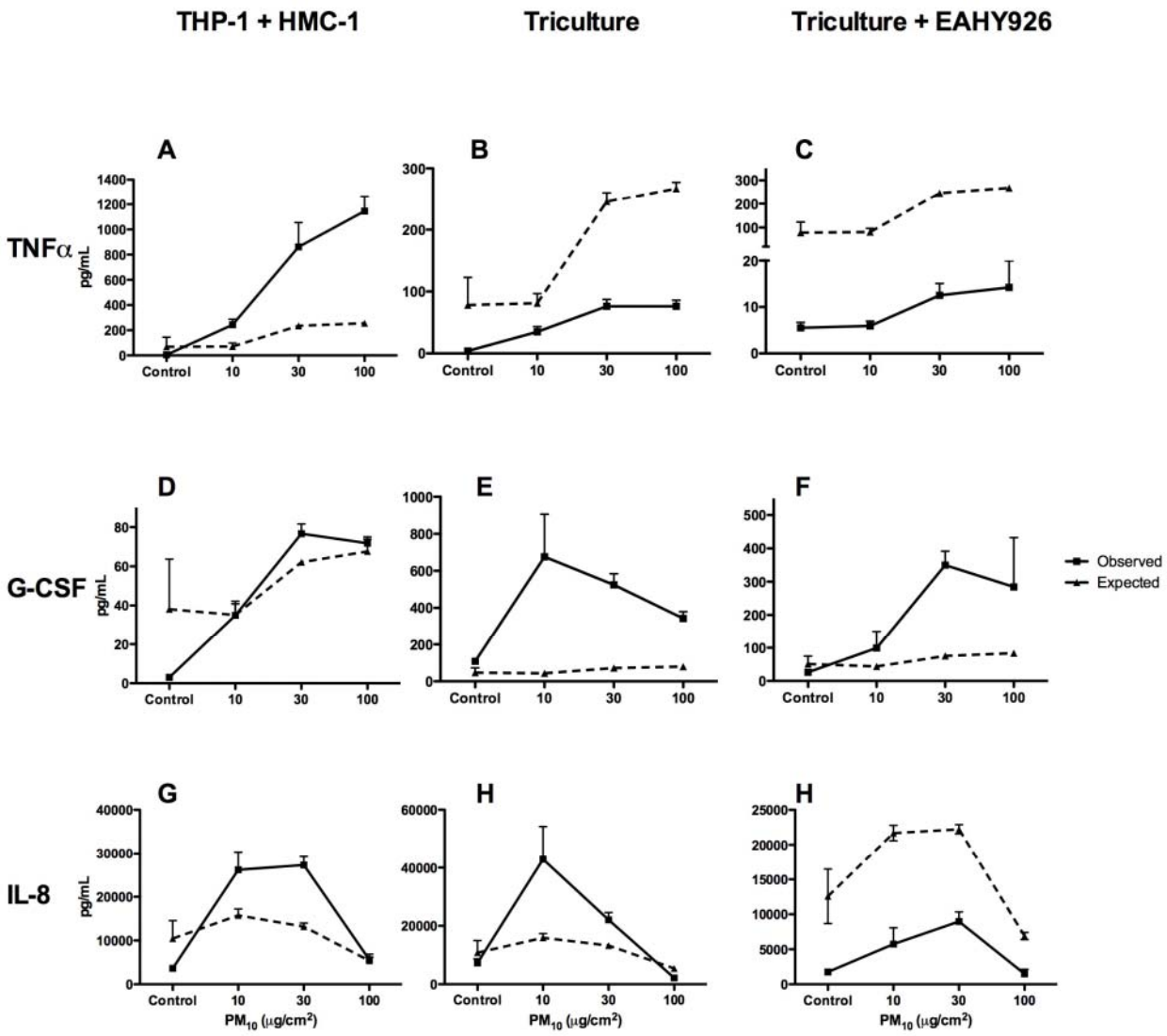
THP-1 + HMC-1
Triculture
Triculture + EAHY926
A
B
C
D
E
F
G
H
H
TNFα
G-CSF
IL-8
pg/mL
Control
10
30
100
PM10 (μg/cm2)
Observed
Expected

Figure 5

Table 1: Medium used for cellular culture.

Cell line	Medium	Other
Single cultures		
A549	DMEM	L-glutamin
THP-1	RPMI	Phorbol myristate acetate (PMA)
HMC-1	Iscove's	α-thioglycerol
EAHY926	DMEM	HEPES
Co-cultures		
A549 + HMC-1	DMEM + Iscove's 10:1	
THP-1 + HMC-1	RPMI + Iscove's 2:1	
TRICULTURE	DMEM + RPMI + Iscove's 10:2:1	
TRICULTURE + Endothelial	TRICULTURE medium + DMEM (HEPES) 3:1	

All the media were supplemented with fetal calf serum (10%), penicillin (100 U/mL), streptomycin (100 µg/mL) and fungizone (1.25 µg/mL). No fetal calf serum was added during exposure to PM_{10}. A549 Lung epithelial type-I like cells; THP-1 Macrophage like cells; HMC-1 Mast cells; EAHY926 Endothelial cells.

Table 2: Semiquantative evaluation of cytokines.

Cytokine	Cell culture				
	A549	THP-1	HMC-1	TRICULTURE	TRICULTURE + Endothelial
G-CSF				x 4.4	x 22.5
GROa	x 0.25				x 0.5
I-309			x 0.35		x 0.07
sICAM		x 2	x 2.7	x 1.5	
IL-1β			x 3.5	x 2.6	
IL-6				x 4.4	
IL-8	x 0.27	x 0.23			
IL-23	x 0.3				
IL-32			x 0.11		
MCP-1			x 0.05		x 0.04
MIP-1α		x 0.5		x 8.4	x 65
MIP-1β		x 1.5		x 13.4	
RANTES		x 1.5		x 0.32	x 0.1
TNFα		x 90			

Mediators presenting relative increases or decreases in the secretion after exposure to PM_{10} (100 $\mu g/cm^2$) are expressed as fold change compared to control, as calculated from the semi-quantitative array membranes (see figure 2). Those mediators with a variation larger than 50% in relation to the control were considered relevant and expressed in this table. A549-Lung epithelial type-I like cells; THP-1 macrophage like cells; HMC-1 Mast cells; EAHY926 Endothelial cells.

Table 3: Overall p-values for percentage changes in cytokines concentrations.

	TNFα	IL-6	IL-1β	IFNγ	G-CSF	IL-8	MCP-1	MIP-1β	MIP-1α	RANTES
A549						<0.001	*<0.001*			<0.001
THP-1	**<0.001**			**0.029**		*<0.055*	0.025			0.016
HMC-1	0.02	0.02					<0.001		<0.001	
EAHY926	**0.01**	0.06			**0.011**					
A549+HMC-1			0.0371			<0.001	<0.001			
THP-1+HMC-1	**<0.001**	**<0.001**	**<0.001**		**<0.001**	*<0.001*	<0.001	**<0.001**	**<0.001**	<0.001
TRICULTURE	**<0.001**	**0.016**	**0.04**		**0.048**	*0.005*	*0.051*	*0.005*	**0.019**	0.024
TRICULTURE+ EAHY926		**0.028**				*0.015*	*0.035*	*0.012*		0.025

Values in bold indicate overall increase of the cytokine; values in plain numbers indicate overall decrease of the cytokine; values in italics indicate increases of the cytokine at low concentrations (10, 30 μg/cm^2) and decreases at high concentrations (100 μg/cm^2). A549-Lung epithelial type-I like cells; THP-1 macrophage like cells; HMC-1 Mast cells; EAHY926 Endothelial cells.